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# $Ca^{2+}$ channel activating action of maitotoxin in cultured brainstem neurons

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**Abstract**

The actions of maitotoxin were studied using cultured brainstem cells and adrenal chromaffin cells. Maitotoxin induced a profound increase in the $Ca^{2+}$ influx into cultured brainstem cells after a brief lag period. The maitotoxin-induced $Ca^{2+}$ influx was suppressed by various voltage-dependent $Ca^{2+}$ channel blockers such as $Co^{2+}$, $Mn^{2+}$, verapamil and diltiazem. Maitotoxin-catecholamine release in brainstem cells initiated to increase after a lag period of about 1 min and the increase continued even at 4 min after treatment, while in the adrenal chromaffin cells the release started after an about 1-min lag period to attain a maximum within first 2-min and gradually decrease thereafter. These results suggest that maitotoxin acts on $Ca^{2+}$ channels to increase the $Ca^{2+}$ influx, accompanied by enhancement of catecholamine release in the brainstem cells with a different temporal profile from that in the adrenal chromaffin cells.


*Keywords:* Maitotoxin; $Ca^{2+}$ channel; Brainstem cell; $Ca^{2+}$ influx; Catecholamine release; Adrenal chromaffin cell

## 1. Introduction

The concentration of intracellular free calcium ($[Ca^{2+}]_i$) is an evolutionarily conserved signal for the regulation of many aspects of cellular function. In both excitable and nonexcitable mammalian cells, $[Ca^{2+}]_i$ plays a crucial role in regulating complex $Ca^{2+}$ signals and differentiated functions, such as excitability, secretion of hormones and release of transmitters, synaptic plasticity, gene expression, and apoptosis (Miller, 1991; Berridge, 1997). The increase in $[Ca^{2+}]_i$ can be due to calcium entry from the extracellular space, through channels in the plasma membrane or from intracellular stores of calcium (mainly endoplasmic reticulm, ER) (Dutta, 2000).

Maitotoxin is a non-peptide toxin produced by toxic, dinoflagellate, *Gambierdiscus toxicus*. A low concentration of maitotoxin induces a profound increase in $Ca^{2+}$ influx into various kinds of cells and initiates a number of $Ca^{2+}$-related cellular functions, including hormonal release from secretory cells, positive inotropic effect on heart, contraction of skeletal and smooth muscles and prolongation of the duration of $Ca^{2+}$-dependent action potential of rat heart and insect skeletal muscles (Miyamoto et al., 1984; Taglialatela et al., 1990; Sauviat, 1997; Obara et al., 1999). Maitotoxin induces $Ca^{2+}$ influx not only into excitable cells but also into non-excitable cells (Gusovsky and Daly, 1990; Columbo et al., 1992; Murata et al., 1992; Watanabe et al., 1993). The toxin per se is unlikely to be an ionophore, so a possibility is that maitotoxin directly stimulates widely distributed $Ca^{2+}$ permiant channels (Takahashi et al., 1983). In neuroblastoma–glioma hybrid cells and certain pituitary tumor cells, maitotoxin-elicited $Ca^{2+}$ influx is blocked at least in part by dihydropyridines that are selective for type L voltage-dependent $Ca^{2+}$ channels (Gusovsky and Daly, 1990). By contrast, in other cell types such as smooth muscle BC3H1 cells (Sladeczek et al., 1988) and synaptosomes (Ueda et al., 1986), these $Ca^{2+}$ channel antagonists have no effect on

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maitotoxin-elicited $Ca^{2+}$ influx. The results demonstrate that maitotoxin can lead to both activation of dihydropyridine-sensitive L-type $Ca^{2+}$ channels and of other $Ca^{2+}$ channels that are not sensitive to dihydropyridines (Gusovsky and Daly, 1990).

In catecholamine-containing tissues, maitotoxin causes the release of catecholamines from PC12 cells and the releasing action is markedly suppressed by various $Ca^{2+}$ channel blockers, such as divalent cations, dihydropyridines, and phenylalkylamines (Takahashi et al., 1983). Maitotoxin also induces the norepinephrine release from adrenergic nerve terminals in guinea pig vas deference (Kobayashi et al., 1985). These results suggest that maitotoxin in part acts on $Ca^{2+}$ channels of various kinds of cells and increases the $Ca^{2+}$ permeability of these cell membranes. In this study, we examined the effect of maitotoxin on the catecholamine release from cultured brainstem cells and adrenal chromaffin cells and compared the action of the toxin on them.

## 2. Materials and methods

### 2.1. Materials

Maitotoxin was extracted and purified as described previously (Takahashi et al., 1982). As for the experiment for cultured bovine adrenal chromaffin cells, maitotoxin was purchased from Wako (Osaka, Japan). Monoclonal antibody to tyrosine hydroxylase (PCTH-7) was kind gift of Dr. Hiroshi Hatanaka. Monoclonal antiglial fibrillary acidic protein was obtained from Amersham (Buckinghamshire). Rhodamine-conjugated goat immunoglobulin G fraction to mouse immunoglobulins, Cappel Laboratories (Cochranville, PA). Dulbecco's modified Eagle's and Ham's F12 media, and horse serum were purchased from Gibco Laboratories (Grand Island, NY). Precolostrum newborn calf serum was obtained from Mitsubishi-Kasei Chemical Industry Co., Ltd. (Tokyo). Trypsin (1:250) was purchased from Difco Laboratories (Detroit, MI). The following materials were obtained from the companies indicated: l-[7-$^{3}$H]norepinephrine (specific activity 46.7 Ci/mmol), [$^{45}$Ca]$Cl_2$ and [$^{22}$Na]Cl, New England Nuclear (Boston, MA); poly-D-lysine (Molecular weight; 30,000–150,000), deoxyribonuclease I and verapamil, Sigma Chemical Co. (St. Louis, MO); tetrodotoxin, Sankyo (Tokyo); diltiazem, Tanabe (Osaka); nicardipine, Yamanouchi (Tokyo): All other chemicals were reagent grade.

### 2.2. Cell culture

Rat brainstem was dissected from 16-day fetal rats (Wistar ST) and was digested with 0.25% (w/v) trypsin and 20 μg/ml deoxyribonuclease I in 1 ml of $Ca^{2+}$ $Mg^{2+}$-free Dulbecco's phosphate-buffered saline (CMF-PBS) for 15 min at 37 °C. Trypsinization was terminated by adding equal volume of heat-inactivated (56 °C, 30 min) horse serum and 10 ml of culture medium. After centrifugation at 150×*g* for 5 min, the tissue was dissociated into single cells by gentle pipetting and then the cell suspension was filtered through a double layer of lend paper. The cells were plated on poly-D-lysine coated 35-mm culture dish at a density of $3.5\times10^{5}$ cells/cm$^{2}$ and the culture were maintained at 37 °C in a $CO_2$ incubator (95% air/5% $CO_2$). Poly-D-lysine coated surfaces were prepared by allowing 1 ml of a solution of poly-D-lysine hydrobromide (0.1 mg/ml water) to stand on culture dishes for 2–3 days at room temperature, after which it was aspirated, the surface was washed twice with water and once with culture medium. The culture medium contained 90% DF medium, 5% precolostrum newborn calf serum and 5% heat-inactivated horse serum. DF medium consisted of 1:1 mixture of Dulbecco's modified Eagle's and Ham's F12 media supplemented with 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesalfonic acid (Hepes) buffer, 30 nM selenium, 50 U/ml penicillin and 200 μg/ml streptomycin. The medium was changed 3 days after cell plating and hereafter every other days. Bovine adrenal glands were kindly provided by the Iwate Chikusan Center. Adrenal chromaffin cells were prepared by collagenase digestion as described previously (Tachikawa et al., 2003). The isolated cells were suspended in Eagle's minimum essential medium containing 10% calf serum, 3 μM cytosine arabinoside, and antibiotics (100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.3 μg/ml of amphotericin B), and were maintained in monolayer culture in 35-mm dishes at a density of $2\times10^{6}$ cells or in 24-well plates at a density of $6\times10^{5}$ cells. The cells were cultured at 37 °C in a $CO_2$ incubator (95% air/5% $CO_2$). Contents of epinephrine, norepinephrine and dopamine in $6\times10^{5}$ cells plated on 24-well plates 101.9±3.5 nmol, 10.9±0.5 nmol and 0.7±0.05 nmol. Additionally, all animal procedures were designed in accordance with the Institutional Guidelines of Tohoku University, Sendai, for the care and use of laboratory animals.

### 2.3. Immunofluorescent staining

After washing with CMF-PBS, brainstem cells on culture dish were fixed in absolute methanol at −20 °C for 20 min and were washed with CMF-PBS (5 min×3) and treated with buffer A (0.05% Triton X-100, 0.01% timerosal and 2% chick serum in CMF-PBS) at room temperature for 20 min. Cells incubated with anti-tyrosine hydroxylase monoclonal antibody (23 μg/ml) in buffer A for 60 min at 37 °C. Cells were washed with buffer A (5 min×3) and incubated with a 1:100 diluted solution of rhodamine–goat–anti mouse immunoglobulins in buffer A (37 °C, 60 min) and after rinsing with buffer A (5 min×3), mounted with a glycerol buffer and examined using a ×40 objective on a Nikon fluorescence microscope.

### 2.4. Assay of $^{45}$Ca influx and [$^{3}$H]norepinephrine release from brainstem cells

$^{45}$Ca influx and [$^{3}$H]norepinephrine release were assayed by essentially the same methods as described before except the amount of [$^{3}$H]norepinephrine was elevated to 4 μCi/dish and that of $^{45}$Ca was increased to 0.5 and 1 μCi/ml for the maitotoxin and high $K^{+}$ induced $^{45}$Ca influx assays, respectively. The assay solution contained 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.87 mM $MgSO_4$, 5.5 mM glucose, and